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Environmental constraints in the study of flexible segments of proteins

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Abstract

The structural problem posed by ill-defined segments in protein structures is similar to those encountered in the study of most peptide hormones, with terminal tracts resembling linear peptides and loops resembling cyclic peptides. The conformational preferences of short linear peptides in solution can be influenced by the use of solvent mixtures of viscosity higher than that of pure water but comparable to that of cytoplasm. In order to check whether it is possible to use these media in the structural study of proteins, we undertook an exploratory study on BPTI in a mixture of dimethylsulfoxide and water. The complete assignment of BPTI in an 80:20 (by volume) DMSO-d_6/water cryomixture at two temperatures showed that all resonances parallel those in water, hinting at the persistence of the correct protein architecture, which is also confirmed by NOESY experiments. In addition to the NOEs present in the aqueous solution it was possible to detect numerous new cross peaks, in particular from residues belonging to the less-defined regions. The new cross peaks do not originate from spin diffusion and are consistent with the best NMR structure and with the X-ray structures of BPTI.

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; BPTI, bovine pancreatic trypsin inhibitor; DMSO-d_6, perdeutero dimethylsulfoxide; DQF-COSY, double quantum filtered correlation spectroscopy; HEWL, hen egg white lysozyme; NOE, nuclear Overhauser enhancement; NOESY, 2D NOE spectroscopy; ROESY, 2D rotational Overhauser enhancement spectroscopy; TOCSY, 2D total correlation spectroscopy.

Introduction

Solution studies of proteins based on NMR spectroscopy have a reliability comparable to that of solid-state studies, but share with diffraction studies the same, minor difficulty. Even in well-refined protein structures there may be ill-defined regions; i.e., owing to their greater flexibility with respect to more structured regions, limited portions of the final structure may be affected by some degree of uncertainty. The less-defined structural elements coincide often with N-terminal or C-terminal segments or with loops connecting regular secondary structure elements (α-helices and β-sheets), but also with several mobile side chains, even of residues that are part of well-defined secondary structures. Flexible loops are often also regions that are involved in molecular recognition and, as such, very interesting from the point of view of structure–activity relationships. It may be argued that they owe their flexibility to the (evolutionary) necessity of adapting their conformation to the shape of the interacting counterpart. It is also true, however, that it is desirable to describe at least the conformational preferences of these tracts with the utmost possible accuracy.

The structural problem posed by these ill-defined segments is similar to those encountered in the study

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of most peptide hormones. Although it is not granted, in principle, that the so-called 'bioactive conformation' of a small peptide can be found among the conformers in equilibrium in solution, it is often found that it is possible to design new agonists or antagonists on the basis of conformational preferences in solution (e.g., Salvadori et al. (1995)). Similarly, the disorder of protein regions may be only partial and may hide valuable structural information, e.g., conformations similar to the interacting conformation, akin to the quoted bioactive conformations of peptide hormones. There are essentially two ways to extract more information from NMR studies of flexible molecules: either introducing further constraints or using sophisticated treatments of the data, e.g., ensemble calculations (Brüschweiler et al., 1991; Amodeo et al., 1992; Blackledge et al., 1993; Nikiforovich et al., 1993; Cicero et al., 1995; Crescenzi et al., 1996). The use of ensemble calculations, in turn, depends on the availability of good NMR data, e.g., a high number of NOEs, possibly more than those observed for the rigid parts if we have to accommodate several conformers in fast equilibrium. The introduction of constitutional constraints in the sequences of flexible regions in the form of more rigid amino acid residues, including some typical non-proteic residues customarily used for this purpose, may lead to more amenable structure determinations but also to undesirable changes of activity and, in many cases, can be utterly impractical. On the other hand, it may be possible to influence the conformational equilibrium of flexible regions by an appropriate choice of the environment, i.e., by the use of what we have termed 'environmental constraints' (Temussi et al., 1992). During the past few years we have shown that the measurement of NOEs and, to some extent, even the conformation of short linear peptides in solution can be influenced by the use of cryoprotective mixtures (Douzou and Petsko, 1984), i.e., solvent mixtures of viscosity higher than that of pure water but comparable to that of cytoplasm (Amodeo et al., 1991). These media can actually play the role of effective environmental constraints since they act as conformational sieves that can select ordered, more compact conformers with respect to extended and/or disordered ones.

Is it possible to use these 'environmental constraints with proteins? It is first necessary to find out whether it is possible to dissolve a globular protein in a cryoprotective mixture without affecting the main elements of secondary and tertiary structure, i.e., its architecture. If this is possible, the more flexible segments may be conformationally constrained by the properties of the medium and thus become amenable to a more detailed structural determination.

We chose BPTI to undertake an exploratory study because of its stability and its paradigmatic role in protein structure determination (Wüthrich, 1986). Another reason was that its structure is better defined than those of most other proteins studied so far, both in the solid state (Brookhaven Protein Databank (PDB) entry name 4PTI (Deisenhofer and Steigemann, 1975); PDB entry name 5PTI (Wlodawer et al., 1984); PDB entry name 6PTI (Wlodawer et al., 1987); PDB entry name 1BPI (Parkin et al., 1995)) and in solution (PDB entry name 1PIT (Berndt et al., 1992)). The definition and quality of the available structures rather than being a drawback render BPTI a convincing benchmark for eventual methodological improvement since they will make it easier to spot even slight deviations from reported structural data. There are hardly regions of BPTI that can be considered 'ill-defined', but it is true that the conformations of the terminal regions and of three specific segments (11–17, 25–30 and 36–39) were defined on the basis of a fairly small number of distance constraints even in the latest, best-refined NMR determination (Berndt et al., 1992).

As high-viscosity medium we chose DMSO-d_6/water since its behavior is better known than that of any other cryomixture (Motta et al., 1987; Amodeo et al., 1991). Here we present the complete assignment of BPTI in an 80:20 (by volume) DMSO-d_6/water cryomixture at 300 K and the detection of new NOEs of the flexible segments arising from conformational sieving.

Materials and methods

BPTI (Aprotinin) was purchased from Fluka AG and used without further purification since analytical reverse-phase chromatography showed a single peak. NMR spectra in water showed its purity to be consistent with published NMR data (Berndt et al., 1992); in particular, the resonance of the aromatic protons of Tyr^{23} is perfectly symmetrical.

NMR measurements

Samples for NMR measurements were prepared by first dissolving the protein in H_2O and adjusting the pH to 5 with HCl. The appropriate amount of D_2O was added for the spectra in water. Increasing amounts of DMSO$-d_6$ (Fluka AG) were added in very small step-

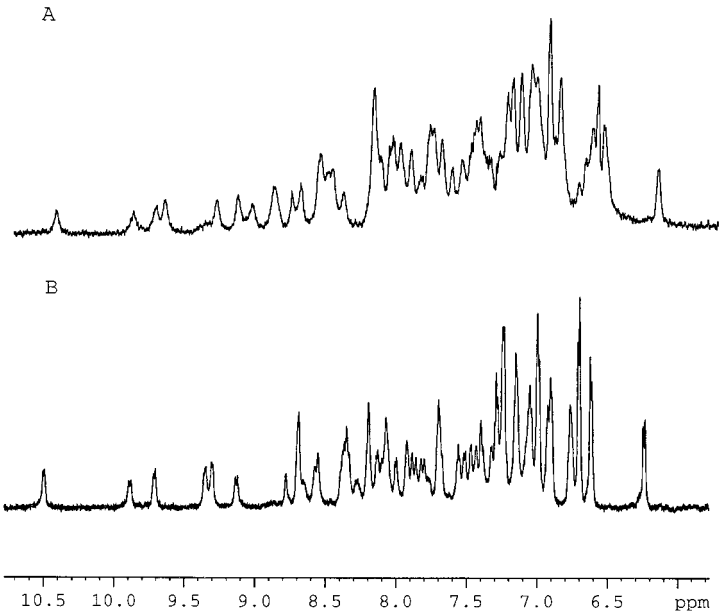

Figure 1. 1D 600 MHz ^{1}H spectra of BPTI in the (A) 80:20 (by volume) DMSO-d_6/water cryomixture and in (B) water at 300 K.

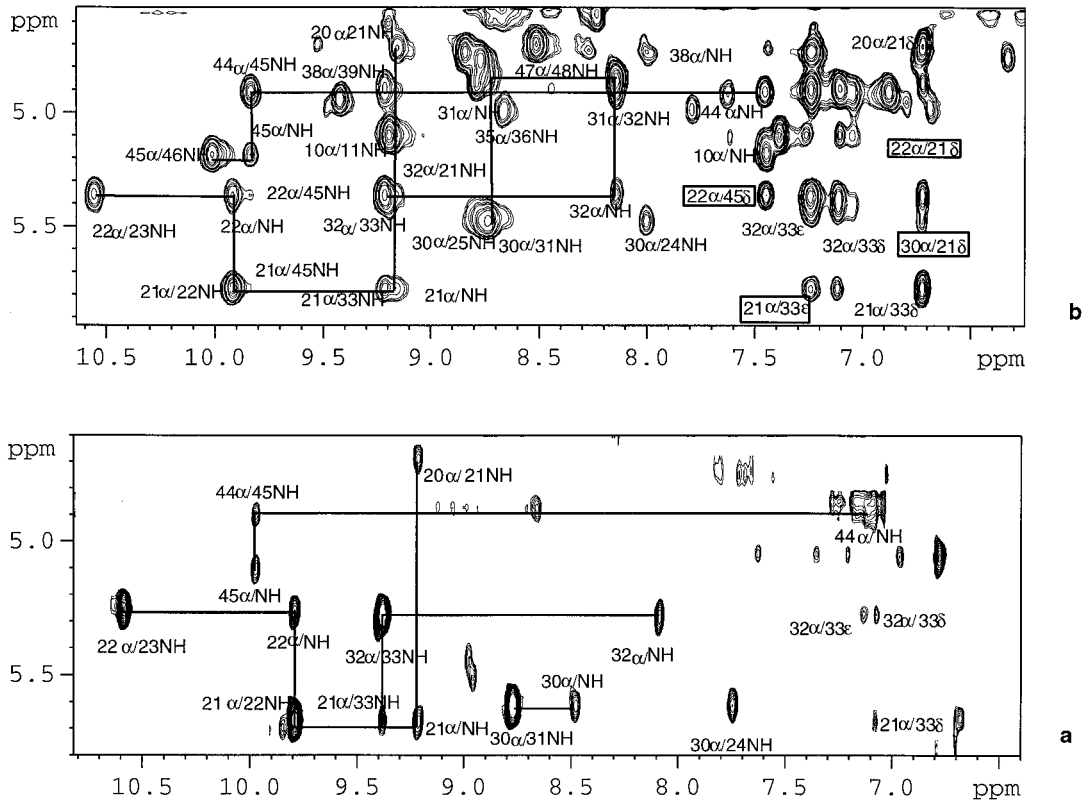

Figure 2. Comparison of a portion of the fingerprint regions of the 600 MHz ^{1}H NOESY spectra of BPTI in water (a) and in the 80:20 (by volume) DMSO-d_6/water cryomixture (b) at 300 K. The mixing times were 120 ms and 50 ms for the spectra in water and in the cryomixture, respectively.

wise increments up to the desired DMSO−d_6/water ratio of 80:20 by volume and a final protein concentration of 10 mg/ml. Since direct dissolution in DMSO-d_6 causes irreversible denaturation, the first sample in the DMSO-d_6/water cryomixture was prepared by titration of a water sample with small volumes of DMSO-d_6. 1D protonic experiments were performed after each DMSO-d_6 addition.

Measurements were performed on Bruker AM-400, AMX-500 and DRX-600 spectrometers in the temperature range 310–278 K. A set of 2D spectra were recorded: COSY (Aue et al., 1976), DQF-COSY (Piantini et al., 1982), TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) (50 ms mixing time) and NOESY (Jeener et al., 1979) (50–125 ms mixing time). TPPI was applied to achieve quadrature detection in the virtual dimension (Marion and Wüthrich, 1983). Water suppression was achieved using WATERGATE (Piotto et al., 1992). The 2D spectra were typically recorded with a spectral width of 8333 Hz; 1024/2048 data points were acquired in t_1/t_2. NOE build-up curves and estimates of correlation times were obtained from a treatment of original NOESY data with AURELIA, release 2.0.

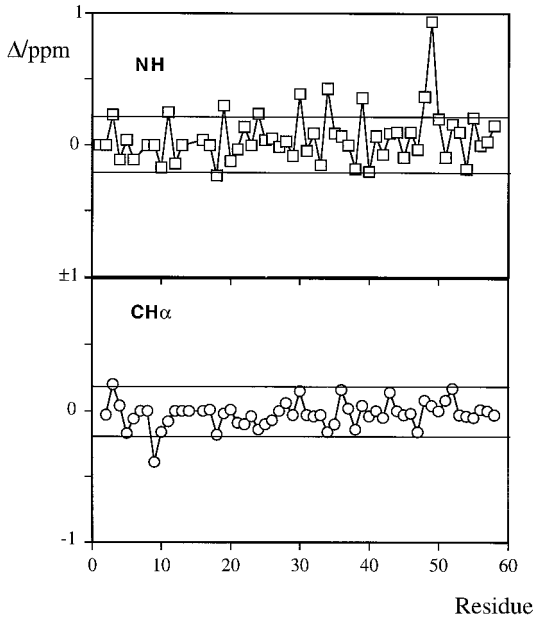

Figure 3. Chemical shift differences between corresponding backbone resonances in water and in the 80:20 (by volume) DMSO-d_6/water cryomixture at 300 K. The chemical shifts in water are referenced with respect to the water resonance (4.70 ppm), whereas those in the cryomixture are referenced to the DMSO peak (2.50 ppm) and then scaled by 0.15 ppm.

Results

NMR assignments

The 1D spectra of BPTI in water and in the 80:20 (by volume) DMSO-d_6/water cryomixture at room temperature (Figure 1) have similar profiles and nearly identical spreads of the NH chemical shifts, but the resonances have rather different line widths. The similarity of the spectra hints that the main elements of secondary and tertiary structure are retained in the mixed solvent, but a more detailed comparison of the spectra is hindered by the substantial increase in line width brought about by the increase of viscosity from 1.0 cp to 3.5 cp and by some accidental superpositions. The assignment of all proton resonances in the 80:20 (by volume) DMSO-d_6/water cryomixture was greatly facilitated by the similarity of the spectra, but was nevertheless performed anew in the conventional manner by the standard protocol (Wüthrich, 1986) based on the use of DQF-COSY, TOCSY and NOESY spectra.

The persistence of the correct protein architecture is confirmed by NOESY experiments and by the detailed comparison of chemical shift differences (vide infra). Figure 2 shows the comparison of a portion of the fingerprint regions of the NOESY spectra of BPTI in water and in the cryomixture. It can be seen that the same sequential paths can be identified both in the aqueous solution and in the cryomixture; the spectrum in the cryomixture, however, shows more peaks.

The complete resonance assignment of BPTI in the 80:20 (by volume) DMSO-d_6/water cryomixture at room temperature shows chemical shifts (referenced to the residual DMSO resonance) superficially different from the corresponding shifts in water (Wagner et al., 1987), but the main contribution to the differences is a uniform high field displacement of 0.15 ppm for all resonances, i.e., a simple scale factor induced by global solvent properties such as bulk susceptibilities. That is, if we add 0.15 ppm to all chemical shifts, the differences between pairs of corresponding chemical shifts are minimal and concentrated in the terminal regions. This view is well illustrated by Figure 3, in which we plotted the differences between corresponding backbone chemical shifts in the two solvents after an appropriate uniform scaling to minimize the influence of bulk solvent properties. It can be seen that most chemical shifts, i.e., over 93%, show differences smaller than ±0.2 ppm. The largest observed difference (0.9 ppm) is that of the NH of residue 49 which is in the C-terminal helix.

The finding that the chemical shifts in the cryomixture are very similar to those in water hints that the architecture of the protein is well preserved in the cryomixture and, very likely, that the protein keeps at least one layer of tightly bound water molecules even in the mixed solvent. This finding is consistent with a recent experimental observation on the specificity of the binding of small organic molecules to HEWL in several mixed aqueous solvents (Liepinsh and Otting, 1997), showing that organic molecules do not compete for bulk solvation but can eventually occupy only specific cavities. It is very interesting to note (Figure 2) that, as we had foreseen, in addition to the NOEs observed in water, the NOESY in the DMSO-d_6/water cryomixture shows several new cross peaks. It is reasonable to assume that the appearance of new cross peaks can be attributed to the increase of viscosity that accompanies the change from pure water to the 80:20 (by volume) DMSO-d_6/water cryomixture at room temperature, i.e., from 1.0 cp to ca. 3.5 cp (Schichman and Amey, 1971).

Environmental constraints

As mentioned in the Introduction section, solvent mixtures of viscosity higher than that of pure water allow the choice of the most convenient correlation time for the measurement of NOEs but can also influence the conformation of short linear peptides, favoring ordered, more compact conformers over extended and/or disordered ones. However, the use of high-viscosity media can broaden protein resonances to a point that makes the spectra very difficult to analyze. It is thus essential to find a good compromise between positive effects (the appearance of new NOEs) and adverse ones (peak broadening) caused by higher viscosity. In order to check the relevance of viscosity and to find optimal experimental conditions we ran NOESY experiments in the 80:20 (by volume) DMSO-d_6/water cryomixture at several temperatures.

Figure 4 shows the comparison between the cross peaks in water at room temperature (300 K) and the corresponding ones in cryomixture at three different temperatures (290, 300 and 310 K) in a small portion of a typical NOESY. The effects reported in Figure 4 originate from the ϵ protons of Tyr^{23}, a residue strategically positioned at the beginning of the loop connecting the strands 18–23 and 31–36. It can be seen from Figure 4 that the number of cross peaks is greatly augmented in going from water to cryomixture at 300 K. The spectrum at 290 K, corresponding to a viscosity of 4.0 cp, shows the same number of peaks as that at 300 K, but line broadening is higher and can adversely affect intensity measurements. On the other hand, the NOESY at 310 K, corresponding to a viscosity of 2.6 cp, shows essentially the same cross peaks observed in water since the new peaks observed at 300 K are now barely detectable. The spectrum in water at 280 K (not shown) is virtually identical to that at 300 K.

The appearance of new cross peaks in the NOESYs in the cryomixture is consistent with the ability of the DMSO-d_6/water mixture to influence conformer distributions in peptide solutions, but the differences among the spectra at 290, 300 and 310 K are larger than expected in a fairly narrow range of viscosities (2.6–4.0 cp). That is, the change of the NOESY spectra as a function of viscosity is surprisingly sharp, hinting at a non-linear dependence of correlation time on viscosity. In particular, the decrease of viscosity from 3.5 cp (at 300 K) to 2.6 cp (at 310 K) seems too modest to explain the virtual disappearance of all the new cross peaks. The sharp dependence of NOESY spectra on viscosity may be a consequence of several concomitant factors, e.g., changes in the structure of water, that, however, are difficult to describe in detail.

A less exciting possibility, with respect to a direct influence of viscosity on conformation, should be taken into account: it is possible that the observation of a greater number of cross peaks may be due to the onset of massive spin diffusion induced by the increase of correlation time. In order to check these possibilities, we evaluated the correlation times of the protein for all solutions and temperatures and looked for signs of spin diffusion. From the NOEs of Tyr^{23} ϵ/δ protons and Pro^{9} $C_\beta H_2$ geminal protons, it is possible to estimate that the correlation time of the whole BPTI molecule, at 300 K, changes from 1.0 ns to 6.9 ns in going from water to the cryomixture, whereas at 290 K and 310 K in the cryomixture it is 7.6 ns and 1.9 ns, respectively. The increment from 1.0 ns to 6.9 ns is twice as large as that expected from the theory of microviscosity (Gierer and Wirtz, 1953) that yields correlation time (τ_c) as a function of molecular volume (V_m), viscosity (η), a microviscosity factor (f_r) and temperature (T). In fact, if one assumes identical (V_m, f_r) coefficients for water and the cryomixture in the formula

$$\tau_c = \eta V_m f_r / kT$$

a value of the order of 3.5 ns would be expected for the cryomixture. The higher value found experimentally (6.9 ns) is consistent with a stronger interaction

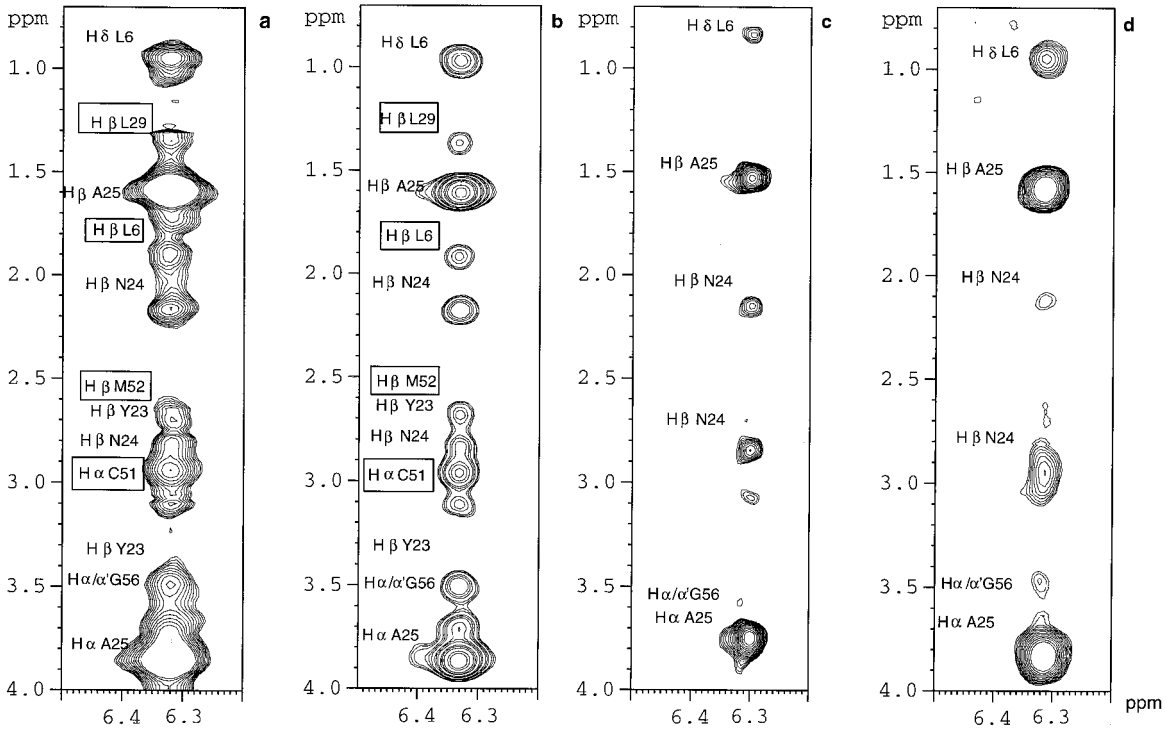

Figure 4. Comparison of a small portion of typical 600 MHz ^{1}H NOESY spectra of BPTI in water (c) at room temperature (300 K) with the corresponding ones in the 80:20 (by volume) DMSO-d_6/water cryomixture at three different temperatures (290, 300 and 310 K, a, b and d respectively).

of BPTI with the mixed solvent, leading to a larger microviscosity factor (f_r) or to a larger apparent molecular volume. Besides, the wider range of correlation times (1.9–7.6 ns) corresponding to the viscosities from 2.6 cp to 4.0 cp is consistent with the very sharp spectral changes described above for the spectra in the cryomixture in the temperature range 290–310 K. The value of τ_c at 300 K is not so large as to necessarily imply a major contribution of spin diffusion for small values of the mixing time; nonetheless, we performed the necessary build-up experiments in the range 0–200 ms of the mixing time of the NOESY experiments at 300 K and checked for the influence of spin diffusion on the intensity of the NOEs. None of the observed cross peaks are significantly affected by spin diffusion. Figure 5 shows representative build-up curves corresponding to some of the NOEs of Figure 4. It can be easily appreciated that the curves of the new peaks observed in the cryomixture (Figure 5, upper graph) are very similar to those of two other cross peaks in water originating from the same diagonal Tyr^{23} ϵ protons (Figure 5, lower graph).

As an indication of the number of cross peaks observed in our media, we have compared the number of effects arising from NH resonances in water and in the DMSOd_6/water mixture. The helical segments showed an overall 41% excess of cross peaks in the cryomixture with respect to water, whereas the 18–35 β-hairpin showed a 49% excess.

A further crucial check of the reliability of the new NOEs may come from their consistency with corresponding distances observed in the solid state (Deisenhofer and Steigemann, 1975; Wlodawer et al., 1984, 1987; Parkin et al., 1995) and in the best-refined NMR structure (Berndt et al., 1992). To choose a meaningful test, we directed our attention only to regions characterized by lower numbers of distance constraints. If one looks at the plot of the number of NOE constraints versus the amino acid sequence of BPTI (Berndt et al., 1992), it is clear that, in addition to the terminal regions, segments 11–17, 25–30 and 36–39, corresponding to the three minima of this plot, are defined by a smaller number of distance constraints. Strictly speaking, these segments are not *ill-defined*, since their conformation is dominated by

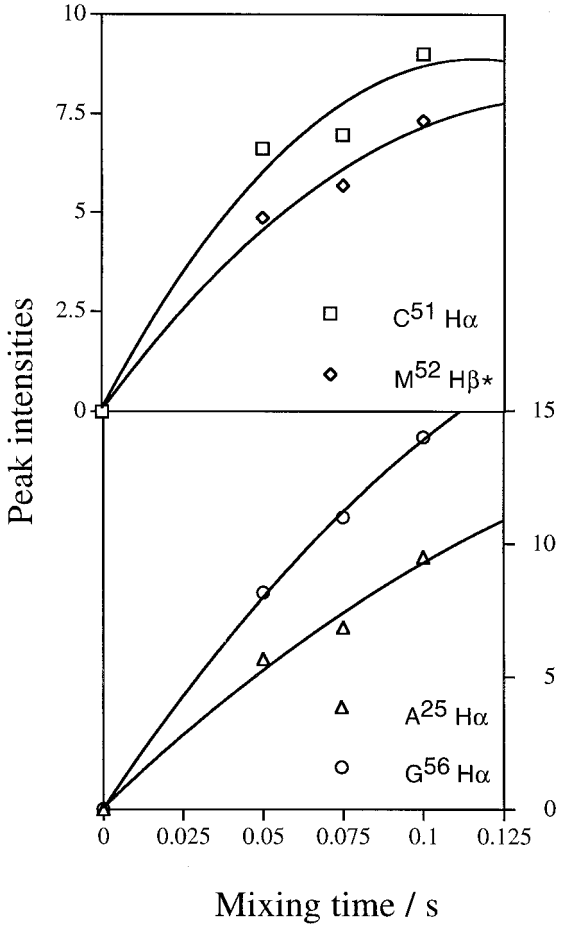

Figure 5. Representative build-up curves of two cross peaks of typical new effects observed in the cryomixture (C51 Hα and M52 Hβ∗, upper graph) and of two cross peaks in water originating from the same diagonal Tyr^{23} ϵ protons (A25 Hα and G56 Hα, lower graph). The range of the mixing times employed in the NOESY experiments at 300 K is 0–200 ms.

Table 1. Comparison of NOE-derived distances of BPTI (residues 23–30) observed in DMSO/water 80/20 at 50 ms with corresponding distances in the X-ray and NMR structures 1BPI and 1PIT

Residue	NOE	Distance (Å)		
		Cryo[a]	1BPI	1PIT
Tyr^{23}	HN F22 HE	3.7	4.50	4.32
	HN N24 HN	3.9	4.41	4.50
	HE K26 HN	4.8	5.80	5.50
	HE A25 HN	4.4	5.0	4.03
	HE G28 HN	4.3	5.30	3.99
	HE L29 HN	4.6	4.40	3.89
	HE C51 HA	4.0	5.80	7.29
	HE M52 HB	5.4	6.18	6.78
	HE L6 HB	5.0	6.60	7.24
	HE L29 HB	5.3	6.14	6.37
Asn^{24}	HN Y23 HN	3.9	4.41	4.50
	HN F22 HE	–[b]	4.40	5.31
Ala^{25}	HN C30 HA	3.8	5.40	7.01
	HN Y23 HE	4.4	5.0	4.03
	HN F22 HE	4.0	4.43	4.76
	HB A27 HN	4.4	5.28	5.07
Lys^{26}	HN Y23 HE	4.8	5.80	5.50
	HN F22 HE	–[b]	5.70	5.68
Ala^{27}	HN A25 HB	5.2	5.28	5.07
Gly^{28}	HN Y23 HE	4.3	5.31	3.99
Leu^{29}	HN Y23 HE	4.6	5.31	3.89
	HB Y23 HE	4.7	6.14	6.37
Cys^{30}	HA A25 HN	–[b]	5.40	7.01
	HN Y21 HE	4.3	5.85	4.44

[a] Distances for groups of protons represent averages.
[b] Superpositions prevent a quantitative estimate.

the overall geometry of the protein, but it is interesting to check whether the use of our environmental constraints yields a larger number of NOEs than in water.

Table 1 shows the comparison of NOE cross-peak intensities for the new peaks originating from protons of residues of the stretch Tyr^{23}-Cys^{30}, as observed in the NOESY of BPTI in an 80:20 (by volume) DMSO-d_6/water cryomixture at room temperature, with corresponding X-ray distances and NMR distances in 1PIT (Berndt et al., 1992). The Tyr^{23}-Cys^{30} sequence embeds residues 25–27 which are the only ones whose geometry in 1PIT is significantly different from that measured in the X-ray structure. Several well-refined solid-state structures are available in the PDB (Deisenhofer and Steigemann, 1975; Wlodawer et al., 1984, 1987; Parkin et al., 1995) but the backbones are very similar. The X-ray distances of Table 1 were taken from the PDB file 1BPI corresponding to a recent solid-state structure (at 0.11 nm resolution) whose data have been collected at a very low temperature (125 K).

It can be appreciated that nearly all new cross peaks correspond to observable distances or to distances that can be reached by modest side-chain rotations. None of the effects reported in Table 1 correspond to distances so long that could not be sampled in a conceivable motion of the protein at room temperature.

Conclusions

The new distance constraints derived from experiments at high viscosity could be used, in principle, for a better definition of the more flexible regions, in conjunction with data collected from the water solution. We did not attempt to recalculate the structures of the more flexible parts of BPTI since calculations of this type are beyond the scope of the present paper.

Acknowledgements

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